Supplementary Materials for

Gut microbiota dysbiosis orchestrates vitiligo-related oxidative stress through the metabolite hippuric acid

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Materials and Methods

Cell culture

All cell lines were cultured at 37 °C in a cell incubator with 5% CO_2. B16F10 cells (kindly provided by Prof. Chunying Li) were maintained in DMEM medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin. All cell lines tested negative for mycoplasma contamination.

Preparation of mouse samples

For tail skin tissue collection, mice were anesthetized with isoflurane (4% for induction and 1.5% for maintenance). Approximately 1 cm of the tail was collected and cut open longitudinally using scissors to obtain tail skin tissue. The collected tissue was either immediately snap-frozen in liquid nitrogen, fixed in formalin or placed in room temperature PBS for further analysis.

For small and large intestine collection, mice were euthanized with an overdose of pentobarbital (250 mg/kg). The abdomen was disinfected with 75% ethanol, and the peritoneal cavity was exposed. Approximately 1.5 cm of the small and large intestines, following the duodenum, were collected and washed in room temperature PBS. The tissues were then fixed in intestine tissue fixative (cat#G1120, Servicebio, China) for subsequent analysis.

Measuring depigmentation

Depigmentation in mice was assessed adapted from Dellacecca *et al.*'s work [19] either before experimental analyses or after euthanasia. Mice were anesthetized with isoflurane (4% for induction and 1.5% for maintenance). When measuring live mice, each experimental cage should be brought to two blinded, trained investigators. Depigmentation was categorized to include both incomplete depigmentation (gray) and complete depigmentation (white). Depigmented areas on the dorsal region, excluding the ears and limbs, were recorded weekly using photographs and quantified with ImageJ v1.54d. Both blinded investigators will subjectively estimate and record the percent depigmentation for each mouse's dorsal region. These individual estimates were then averaged to obtain a final value for each mouse.

H&E staining

Fixed small and large intestinal tissues were dehydrated using a graded ethanol series. Subsequently, these tissues were embedded in paraffin and sectioned into 4 μm thick slices. The sections were stained with haematoxylin and eosin (H&E) using protocols provided by Servicebio (Wuhan, China). The H&E-stained specimens were scanned with Panoramic MIDI and Digital Pathology System (3D Histech, Hungary), and the thickness of the intestinal mucosal layer was assessed based on the observed characteristics of these specimens.

AB staining

Tissue sections were stained with Alcian blue (AB) staining solution (pH 2.5, cat#G1027, Servicebio, China) for 5-10 minutes, followed by rinsing with water.

After dehydration, dewaxing, and sealing, the sections were scanned with Pannoramic MIDI and Digital Pathology System (3D HISTECH, Hungary) to quantify the number of goblet cells per unit length.

IHC analysis

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were sectioned at 5 μm thickness. Paraffin removal was achieved using xylene. For antigen retrieval, deparaffinized and rehydrated sections were immersed in 0.01 M sodium citrate buffer (pH 6.0) and subjected to high-pressure treatment for 3 minutes. Following antigen retrieval, the slides were incubated with the primary antibody (recombinant anti- zonula occludens-1, dilution: 1:1000, cat#GB151981, Servicebio, China; anti-mouse occludin, dilution: 1:1000, cat#GB111401, Servicebio, China) for 4 hours at room temperature, washed with PBS. Detection of the antibodies was carried out using a DAB staining kit (cat#G1212, Servicebio, China). Hematoxylin served as a counterstain, and the slides were mounted with neutral balsam and coverslips. Light microscopy images of the stained sections were captured using Panoramic MIDI and Digital Pathology System (3D HISTECH, Hungary).

The H-scoring system was used to evaluate zonula occludens-1 (ZO-1) and Occludin staining results[43]. The H-score was calculated by multiplying the total staining intensity of each section by the percentage of positive cells. The staining intensity was categorized into four levels: 0 (negative), 1 (weak), 2 (medium), and 3 (strong), with the percentage of positive cells ranging from 0 to 100%. Thus, the final H-score ranged from 0 to 300. We stained ZO-1 and Occludin and determined their values using this scoring system.

Elisa

Serum IL-1β (cat#88-7261, Thermofisher, USA) and 8-OHdG (cat#CEA660Ge, Cloud-Clone Corp, USA) levels were determined via Elisa. The results showed that serum IL-1β levels were significantly higher in the vitiligo group than in the controls, with a *P*-value of 0.016, consistent with previously reported results. Similarly, serum 8-OHdG levels were significantly higher in the vitiligo group than in the controls, with a *P*-value of 0.026, also in agreement with previous findings. Detailed statistical data can be found in table S6.

Transmission electron microscope (TEM)

To prepare tissues for TEM analysis, fresh tissues were harvested within 1-3 minutes using a sharp blade to minimize mechanical damage. Tissue blocks, no larger than 1 mm^3, were placed in petri dishes with TEM fixative (cat#G1124, Servicebio, China) and then transferred to Eppendorf tubes with fresh fixative, stored at 4℃. The tissues were washed with 0.1 M PBS (pH 7.4) three times for 15 minutes each. Post-fixation involved treating tissues with 1% OsO4 in 0.1 M PB (pH 7.4) for 2 hours at room temperature, followed by rinsing in 0.1 M PB (pH 7.4) three times. Dehydration was performed at room temperature using graded ethanol, two changes of 100% ethanol for 20 minutes each, and two changes of acetone for 15 minutes each. For resin embedding, tissues were treated with acetone EMBed 812 mixtures (1:1 for 2-4 hours,

1:2 overnight, and pure EMBed 812 for 5-8 hours) at 37℃. Polymerization occurred at 65℃ for over 48 hours. Ultrathin sections (60-80 nm) were cut, placed on 150-mesh copper grids with formvar film, and stained with 2% uranium acetate for 8 minutes, rinsed with 70% ethanol and ultrapure water, followed by staining with 2.6% lead citrate for 8 minutes. The sections were dried overnight at room temperature and observed under a TEM (HITACHI-HT7800, Japan).

Whole-mount immunofluorescence staining

The procedure for whole-mount immunofluorescence staining was adapted from Xu *et al.* [22] In brief, hair shafts that would interfere with the immunofluorescent imaging process were removed using Nair treatment. The entire tail skin was then collected and flattened and was cut into 1 × 1 cm squares and placed in 20 mM EDTA (cat#E1170, Solarbio) solution for dermal separation. Then occasional hair follicles were removed using tweezers. The epidermis samples were then flattened and fixed for 10 minutes in 4% paraformaldehyde in PBS, washed in PBS overnight and then permeabilized for 20 minutes in 0.3% H_2O_2 in methanol at −20 °C. Samples were blocked for 1 hour in a solution of 2% normal donkey serum, 1% BSA, and 0.3% Triton in PBS at room temperature. The following antibodies were used: anti-mouse melan-A (cat#ab210546, dilution: 1:500, Abcam) and anti-mouse CD8a (cat#14-0808-82, dilution: 1:300, Invitrogen). Whole-mount and section samples were imaged using the FV3000 confocal laser scanning microscope (Olympus, Japan). Microscopy data were analyzed using ImageJ software (version 1.54d).

16S rDNA sequencing and bioinformatic analysis

Fecal content samples were collected into 2 ml sterile cryopreservation tubes and stored at -80°C. DNA was extracted from 200 mg samples using the QIAamp DNA Stool Mini Kit (cat#51604, QIAGEN, Germany) following the manufacturer's instructions. The V3-V4 region of 16S rRNA genes was amplified using the 357F (5'-ACTCCTACGGRAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers on a thermocycler PCR system (LC-BioTechnology Co., Ltd, Hangzhou, China). The PCR products were confirmed via 2% agarose gel electrophoresis, purified using a DNA gel extraction kit (cat#AP-GX-50, Axygen, USA), and quantified using the FTC-3000TM real-time PCR system (Shanghai). The sequencing library was purified using a DNA gel extraction kit (Axygen, USA) and sequenced using 2×250 bp paired-end sequencing on the Novaseq platform with the Novaseq 6000 SP 500 Cycle Reagent Kit (cat#20028402, Illumina, USA) at TinyGen Bio-Tech (Shanghai) Co., Ltd. The 16S sequences were analyzed using a combination of software including Mothur (version 1.33.3), UPARSE (usearch version v8.1.1756), QIIME2 (version 2020.8.0), and R (version 3.6.3). The demultiplexed reads were analyzed using DADA2 (version 2020.8.0) in QIIME2 (version 2020.8.0) for amplicon sequence variant (ASV) analysis, and singleton ASVs were deleted. The ASV representative sequences were assigned for taxonomy against the Silva 128 database with a confidence score ≥ 0.6 using the classify.seqs command in Mothur (version 1.39.5).

For alpha-diversity analysis, the Shannon index and rarefaction curves (not shown) were calculated and plotted using R. For beta-diversity metrics, the unweighted UniFrac distance matrix was calculated and visualized with Principal Coordinate Analysis (PCoA) using the ape package in R. LEfSe analysis was used to identify taxa significantly enriched between the vitiligo and non-induction groups, using a per-sample normalization value of 1,000,000 and default values for other parameters. In LEfSe analysis, the linear discriminant analysis (LDA) score was computed for taxa differentially abundant between the two groups. A taxon with $P < 0.05$ (Kruskal–Walli's test) and log10[LDA] ≥ 2.0 (or ≤ -2.0) was considered significant.

Targeted quantitative metabolomics

Targeted metabolomics profiling was conducted using the Q300 Metabolite Array Kit from Metabo-Profile Biotechnology of China [44]. In brief, metabolites were extracted from lyophilized feces by preparing a homogenate using approximately 5 mg of feces with 25 μL of water. The sample was homogenized with zirconium oxide beads for 3 minutes, and 120 μL of methanol containing an internal standard was added to extract the metabolites. The sample was homogenized for another 3 minutes and then centrifuged at 18,000 g for 20 minutes. Twenty microliters of the supernatant were transferred to a 96-well plate. Subsequent procedures were performed on an Eppendorf epMotion Workstation (Eppendorf Inc., Germany), where 20 μL of freshly prepared derivative reagents was added to each well.

For skin tissue, each tissue sample (~10 mg) was harvested and stored in an Eppendorf Safelock microcentrifuge tube, mixed with 10 pre-chilled zirconium oxide beads and 20 μL of deionized water. The sample was homogenized for 3 minutes, and 120 μL of methanol containing an internal standard was added to extract the metabolites. The sample was homogenized for another 3 minutes and then centrifuged at 18,000 g for 20 minutes. The supernatant was transferred to a 96-well plate, and subsequent procedures were performed on an Eppendorf epMotion Workstation, where 20 μL of freshly prepared derivative reagents was added to each well.

For the serum samples, 20 μL of serum was added to a 96-well plate, which was then transferred to the Eppendorf epMotion Workstation. 120 μL of ice-cold methanol with partial internal standards was automatically added to each sample and vortexed vigorously for 5 minutes. The plate was centrifuged at 4000 g for 30 minutes (Allegra X-15R, Beckman Coulter, USA). The plate was then returned to the workstation, and 30 μL of the supernatant was transferred to a clean 96-well plate, where 20 μL of freshly prepared derivative reagents was added to each well.

The derivatized samples with added internal standards were randomly analyzed and quantified using an ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., USA). A total of 306 standard substances, including 15 subclasses, were obtained from Sigma-Aldrich, Steraloids Inc, and TRC Chemicals. To ensure the quality of the metabolomics platform, three types of quality control samples were routinely used: test mixtures, internal standards, and pooled biological samples. The derivatized

pooled quality control samples were injected. The raw data generated by UPLC-MS/MS were processed using TMBQ software (v1.0, Metabo-Profile, China) to perform peak integration, calibration, and quantitation for each metabolite. The self-developed platform iMAP (v1.0, Metabo-Profile, China) was used for statistical analyses, including PCA, OPLS-DA, univariate analysis, and pathway analysis. Through mass spectrometry-based quantitative metabolomics, metabolomic features were annotated to metabolites with level 1 of confidence by comparing them to the standard metabolites.

Cocktail antibiotic treatment

Mice were treated for 2 weeks with a broad-spectrum antibiotic (Abx) cocktail, comprising ampicillin (1 g/L), metronidazole (1 g/L), neomycin (1 g/L), and vancomycin (1 g/L). This treatment regimen has been extensively proven to effectively deplete indigenous intestinal microbiota and facilitate the colonization of donor-derived gut bacteria. Fecal samples were collected before and after antibiotic treatment to verify the effects (Table S2).

Co-housing experiment

Five 2-month-old vitiligo-induced mice were co-housed with five Day 35 vitiligo-induced mice per cage. Photographs were taken weekly to assess and measure depigmentation in the dorsal skin.

Fecal microbiota transplantation (FMT)

A fecal slurry was prepared by pooling fecal pellets from six to eight donor mice (two fresh fecal pellets per mouse). The fecal pellets were resuspended in 600 μl of reduced PBS (PBS with 0.5 g/L cysteine and 0.2 g/L Na_2S) using a vortex and then filtered through a 70 μm cell strainer to remove insoluble material. Approximately 100 μl of the supernatant was administered to each mouse by oral gavage three times a week for 2 weeks. After this 2-week period, mice received the microbiota suspension once a week until natural death or sacrifice. Fecal samples were collected on Day 35 of the vitiligo induction procedure and stored at −80 °C for 16S rRNA gene sequencing.

Supplementation with probiotics

Probiotics were administered using the compound preparation BIFICO® (cat#S10970105, China), which is approved by the China National Medical Products Administration for clinical use. BIFICO® contains *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*. The probiotic groups (n = 5 mice/group) were orally fed with BIFICO® (1×10^7 CFU) once a day for 4 weeks.

Supplementation with metabolites

Non-induction and vitiligo mice were treated intraperitoneally with 2-phenylpropionate (2-PP), hippuric acid (HA), or indoleacetic acid (IAA) daily from Day 12 to Day 35 during the vitiligo induction procedure. HA was administered via intraperitoneal injection (i.p.) at 3 mg/kg daily (cat#H810748, Macklin), based on previously published data [45] and the conversion of bioavailability from oral gavage

to i.p. Similarly, the i.p. dosage of IAA (cat#12886, Sigma-Aldrich) and 2-PP (cat#P31701, Sigma-Aldrich) was set at 50 mg/kg daily [46].

In vivo intestinal permeability test

To assess in vivo intestinal permeability, tracer fluorescein isothiocyanate (FITC)-labeled dextran (4 kDa; cat#46944, Sigma-Aldrich) was used. Briefly, mice were deprived of food 4 h before and both food and water 4 h after oral gavage using 200 μl of 80 mg/ml FITC-dextran. Blood was collected after 4 h and fluorescence intensity was measured on fluorescence plates using an excitation wavelength of 493 nm and an emission wavelength of 518 nm. To assess in vivo intestinal permeability to hippuric acid, mice were treated with 3 mg/kg hippuric acid. Blood was collected immediately before and 4 h after treatment. Hippuric acid translocated into the circulation was measured by LC–MS.

Reverse virtual screening calculation and bioinformatic analysis

The sdf. format structure file of the small molecule compound was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The three-dimensional structure of the ligand was constructed using ChemBioOffice and optimized through automatic geometric optimization. This optimized three-dimensional structure was then used for reverse virtual screening. A disease target database containing more than 600 targets and over 7000 active sites was selected for the screening. The whole target library included the crystal structures of targets corresponding to disease genes, comprising 2119 active sites of 140 targets. The part target library included target structures for disease genes, comprising 5487 active sites of 506 targets. The high-performance reverse virtual screening platform was used to submit the screening task. Ligand molecules were docked to the binding sites of the proteins in the receptor library one by one. The scoring was based on the binding energy between the ligand and the receptor, and the top 500 results were selected for further analysis. Hippuric acid was evaluated based on binding energy, and the top 500 results were retained.

To further investigate the molecular mechanisms underlying vitiligo, a protein-protein interaction (PPI) network was constructed. Common genes were imported into the STRING database [47] (https://string-db.org/) with the organism limited to "Homo sapiens," setting the minimum required interaction score to the highest confidence (0.9) and hiding disconnected nodes. The PPI network visualization, analysis and calculation of the degree value of nodes also in the STRING database. Gene Ontology (GO) and KEGG pathway enrichment analyses were performed on the core genes of the PPI network. Pathways with a P-value < 0.05 were considered significantly enriched.

Differential Scanning Fluorimetry (DSF)

For the DSF experiments, 20 μL samples were prepared in duplicate using 4 μM of protein (NOS2, cat#TP311819, ORIGENE; MPO, cat#11917-H08H, Sino Biological Inc.; MAPK14, cat#CSB-EP013453Huc0, Cusabio) and hippuric acid (cat#H810748, Macklin) at concentrations ranging from 0 to 500 μM. The samples were heated from 25 to 95 °C with increments of 1 °C/min, followed by a 10-minute incubation period.

Fluorescence was measured at each step using a real-time PCR system (Bio-Rad CFX Opus96, USA). The change in melting temperature (T_m) values was calculated and recorded by the instrument. Data analysis and image generation were performed using GraphPad Prism.

Surface Plasmon Resonance (SPR)

SPR experiments were performed using a Biacore 8k instrument (Cytiva, Sweden). The proteins (Mapk14, MPO, NOS2) were diluted to a concentration of 40 μg/mL and immobilized on a CM5 Series S sensor chip (Cytiva, Sweden) via amine coupling, achieving approximately 21,100 RUs for Mapk14, 15,700 RUs for MPO, and 16,900 RUs for NOS2. Increasing concentrations of hippuric acid were injected at a flow rate of 50 μL/min. The experiments were conducted at 25 °C, with a blank activated/deactivated flow cell serving as a reference. For MPO, the signal did not show a concentration-dependent relationship with hippuric acid, and a saturation curve could not be fitted. This may be due to insufficient protein coupling levels, resulting in undetectable binding signals. Data analysis was performed using Biacore 8k Manager software.